It has been shown previously in this laboratory that immunisation of BALB/c J mice with live Stm as against heat killed preparation of Stm, results in the differential induction of T helper responses. Present study has attempted to resolve some of the issues arising out of this. Splenocytes from the mice that have been immunized with live Stm secrete IFN-\(\gamma\) upon activation and the sera collected from these mice show higher levels of Stm specific IgG2a as against IgG1. These mice also develop higher DTH when tested with sonicated extract of Stm754, which is the candidate strain used. On the other hand, splenocytes from heat killed mice that have been activated with sonicated extract show abundance of IL-10 over IFN-\(\gamma\), develop low DTH and show relatively higher levels of IgG1 in sera as seen by higher ratios of IgG1/IgG2a (Thatte, et al. 1993).

This kind of polarization of T helper responses has been shown in other infection models like Leishmania, Listeria and Trichinella infection in mice. In the case of Leishmania infection, BALB/c J is sensitive to infection and develops Th2 response, whereas, C57BL/6 J is resistant to infection and develops Th1 response against Leishmania. Lot of research work in the last decade was focused on showing the phenomenon of T helper cell skewing in various infection models and now it is time to think about what are the possible factors behind this effect.

Using the novel model system of Stm immunisation, we attempted to dissect out few points that may have relevance as the cause of starting the cascade towards Th1 development. We have probed the factors from the angle of the host, viz. the genetic susceptibility of mice to Stm infection, and from the angle of the pathogen, viz. its ability of it to survive in the host.
Earlier in this laboratory, the regimen of immunisation with live and heat killed Stm followed was, two doses of either live or heat killed Stm. Live immunisation was done with ~10^2 live Stm, followed by ~10^3 bacteria, a week later. Heat-killed immunisation was done with ~10^8 heat-killed bacteria, a week apart. This regimen appeared unnecessarily prolonged, and ways to simplify it were sought. We have developed a regimen, in which we give a single dose of ~10^3 live or ~10^8 heat-killed bacteria. It can be seen from Fig. 1a that the DTH reaction developed after such immunisation, follows the same pattern as seen earlier. It can also be seen from Fig. 1b that the levels of IFN-γ after live Stm immunisation, are abundant as compared to immunization with heat-killed Stm, whereas, immunisation with heat-killed Stm generated relative abundance of IL-10 over IFN-γ. Both these results suggested to us that we could use this simplified regimen of immunisation to probe other factors that may have relevance in developing this phenomenon of polarized Th1-like response after live Stm immunisation.

It has been shown before that anti-class II antibodies block the proliferation of Stm specific T cells from mice that are immunized with live or heat-killed Stm, whereas, anti-class I antibody do not block it appreciably. We have made use of TAP1 gene targeted mouse strain. This strain does not express surface H-2K^b, H-2D^b molecules owing to the defect in peptide fragment transportation to MHC class I molecules to stabilize them. There is a major reduction in terminally glycosylated MHC class I molecules, indicating that the immature, empty molecules accumulate in endoplasmic reticulum. This gene targeted mouse lacks CD8^+CD4^- T cells as their selection takes place only minimally in the absence of class I molecules (Van Kaer L, et al. 1992). It has been shown in the case of live Listeria infection, that because of listeriolysin O, holes in the phagosomes are created (Camilli A, et al. 1989), which makes the parasite
leach into cytosol where they grow very freely. This has been shown to help the presentation of parasite peptides in the context of class I MHC molecules and hence generating the CD8 T cells against *Listeria* peptides (Darji A, *et al.* 1995) including an immunodominant epitope from listeriolysin O (Hiltbold EM, *et al.* 1996). It remained to be seen whether CD8 T cells are contributing significantly in immunity towards Stm. We have tried to ask this question by using these *TAP1* gene targeted mice defective in CD8 T cell response. From Fig. 3 the minimal involvement of CD8 T cells is seen by the amounts of cytokines secreted by *TAP1* gene targeted mice and C57BL/6 J strain of mice after immunisation with live or heat-killed immunisation. It can be seen from Fig. 3c that overall ratios of IL-10/IFN-γ are unaltered when *TAP1* deficient strain is compared to C57BL/6 J strain. As expected, live and heat-killed immunisation show lower ratios as compared to heat-killed immunisation owing to higher IFN-γ levels.

In many other parasitic infections, the presentation of parasite peptides are in the context of class II MHC molecules by APCs and thereby generate CD4 T cell help. Intracellular facultative parasites like leishmania, mycobacteria shed their peptides in the phagosomes where they are loaded on to class II MHC molecules (Scott P & Kaufmann SHE. 1991). The extent of involvement of MHC class II molecules is seen in present study using gene targeted mice for I-Aβα. MHC class II gene targeted mice do not develop mature single positive CD4 T cells in periphery. As seen from Fig. 4, virtually all the response that is observed in C57BL/6 J strain disappears in MHC class II gene targeted strain thereby providing evidence of its involvement. The response is not abolished completely. The remainder of response may be due to the class I presentation of peptides or due to the involvement of non-classical MHC molecules. It has been shown in the case of *Mycobacterium*, that lipid antigens are presented by
non-classical MHC molecule, CD1, to generate T cell help (Beckman EM, et al. 1994). Similarly in another case, mycobacterial macromolecular complex are presented via CD1b, a nonclassical MHC molecule, to CD4"^"CD8"^" T cells (Porcelli S, et al. 1992). Apart from these examples, there are numerous reports suggesting that bacterial components like carbohydrates, lipid, glycopeptides and peptidoglycans are presented on non-classical MHC molecules (Porcelli A, et al. 1996), including the n-formylated peptides by H2-M3 (Lenz LL, et al. 1996). This may be true for Stm, since it has many other macromolecular components apart from proteins e.g. lipophosphoglycans. Even though LPS has been removed from Stm sonicate by ultracentrifugation at 100,000 \( g_{\text{max}} \) for 1 hr., remaining traces may be enough mediate such a response.

**Protein modification in heat killed versus live Stm**

In all the assays described to determine the cytokine secretion pattern from the splenocytes of the mice immunized with live and heat killed Stm, we have used the soluble protein extract prepared from the heat-killed Stm. It is quite possible that the heat treatment of the soluble protein extract (and hence heat-killed Stm) may abrogate their ability to prime towards Th1 pattern. As has been shown, mice immunized with either sonicate prepared from heat-killed or live Stm show the same pattern of cytokine expression (Fig.5).

**Stress related proteins**

From the literature, evidently, there is a certain defined cascade of events that are common to all Th1 developments. It starts with IL-12 production by macrophages and NK cells, which subsequently drive IFN-\( \gamma \) production that
then helps in the development of Th1-like response. Studies to this end were also done initially by Gajewski et al. (1989), where they pointed out that administration of the neutralizing antibodies to IFN-γ at the time of priming, leads to a Th2-like response to the antigen in question. What factors trigger the start of this cascade is a point to ponder. Stm is known to start transcribing a variety of proteins upon entering macrophages. After the infection of macrophages with Stm, bacteria start expressing stress related proteins (Buchmeier and Heffron, 1990). Moreover, it is shown that once they are into phago-lysosomes, due to the distinct environmental signals, Stm start expressing distinct proteins that are useful in its virulence (Mekalanos JJ, 1992) some of these genes are regulated by as many as ~18 pH related operons which are turned on after encountering low pH (Foster JW, et al. 1994), while some more are regulated by PhoPQ regulon (Vescovi GE, et al. 1994). All these stress related genes are absent in the heat-killed preparation of Stm. Although, the transcriptional activity in Stm metabolic mutants discussed above may be minimal, it can not be ruled out completely. Small amount of these proteins may be responsible for immunisation. Thus, some of these protein products may be responsible for generating a strong Th1-like response after live immunisation with Stm or its metabolic mutants but not in heat-killed or transcriptionally blocked (irradiated bacteria) Stm. It has similarly been reported that bacterial DNA induces NK cell mediated IFN-γ and other pro-inflammatory cytokines (Cowdery JS, et al. 1996), which obviously heat-killed Stm will not be able to induce because of the destruction of DNA at very high temperature of the killing procedure.
**Host defense of Infection and its effect on T helper outcome: Ity locus**

The development of Th1-like response in live Stm immunisation was noted in earlier studies, whereas, heat-killed immunisation developed Th2-like T helper response. It has been reviewed earlier that, other mouse infection models of intracellular parasites, also show Th1-like response after live immunisation. Whether this was due to the exclusive property of these bacteria to replicate and persist inside the macrophages, remained to be elucidated.

*Ity* locus has been shown before to restrict the growth of intracellularly replicating Stm, *Leishmania*, and *Mycobacterium bovis* (Vidal S, et al. 1995). It has been shown that the product of *Nramp1* gene is a transmembrane protein, which has the properties of ion channel proteins. This gene product in *Ity* mice probably transports reactive nitrogen radicals which are crucial for microbicidal action of macrophages; also macrophages in *Ity* mice are in a more activated state than those from *Ity* mice. Immunisation with live Stm in *Ity* and *Ity* mice raises two points; whether there are effects on quantitative and/or qualitative immune response in terms of T helper cell development.

We have used two such pairs of mice having either H-2d (BALB/c J & C.D2) or H-2b (C57BL6/J & C3H.SW) haplotypes. C.D2 and C3H.SW are *Ity* strains. As it can be seen from Fig.6, Stm growth is restricted in C.D2 mice. At all the time points checked in this assay, CFU of Stm is always ten fold less in *Ity*. Less number of continually growing bacteria however did not show any alteration in cytokine pattern of T helper response generated to live or heat-killed Stm. Small number of live bacteria in *Ity* mice, at earlier time points are hence probably enough to prime the T cells of the mice to Th1-like response after live Stm immunisation.
It has been demonstrated earlier that, upon infection with Stm, \textit{Ity}^f mice produce significantly higher numbers of \(\gamma\delta\) T cells (Emoto \textit{et al.} 1993). Some studies also document that \textit{Ity}^f mice produce copious amounts of NK cell- & \(\gamma\delta\) T cell mediated IFN-\(\gamma\) (Ramarathinam \textit{L et al.} 1993) as well as TNF-\(\alpha\) and IL-1 (Formica \textit{S et al.} 1994; Kita \textit{E et al.} 1992) at earlier time point after infection with live Stm. According to Gajewsky \textit{et al.} (1989), production of IFN-\(\gamma\) at earlier time points of T cell priming may lead to enhanced Th1-like response. Contrary to the report of increased IFN-\(\gamma\) in \textit{Ity}^f, it has been demonstrated that \textit{Ity}^s mice are not innately defective in IFN-\(\gamma\) production at earlier time points as compared to \textit{Ity}^f mice; their susceptibility is attributed to the fact that IL-10 is expressed in significantly higher amounts in \textit{Ity}^s strains as compared to \textit{Ity}^f strains (Pie \textit{S et al.} 1996). This differential cytokine scenario probably does not then play a significant role in shaping the T helper differentiation. The fact that Th1-like response is seen in \textit{Ity}^f mice, where Stm are cleared tenfold better, may be due to an interplay between the quantitatively less persistence of bacteria and enhanced IFN-\(\gamma\) production showing the net result of Th1-like response development.

It should be noted that less persistence of Stm in \textit{Ity}^f mice is due to the enhanced killing ability of macrophages in these strains. Stm, however, can get inside the macrophages of \textit{Ity}^f strains as actively as it can in \textit{Ity}^s mice as entry is not affected by \textit{Nramp1} gene product. It has been shown by electron microscopy that live Stm prevents phagolysosomal fusion once inside the macrophages. This is not shown to be the case when macrophages are pulsed with heat-killed or gluteraldehyde fixed Stm in the same experiment (Buchmeier \textit{N and Heffron F}, 1991). It is possible that by virtue of entering phagosomes, the processing and presentation of the immunodominant peptides
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generated from live Stm are qualitatively different than those generated after immunisation with heat-killed immunisation. Alternatively, heat shock proteins (HSPs) generated during the heat killing procedure may be immunodominant and develop Th2-like immune response resulting in the distinct differences in immunisation with live and heat killed Stm. In the previous studies (Thatte J, Ph.D. Thesis), experiments were done to this end to check the possible distinctly dominant proteins, against which the response is mounted after immunisation with live and heat killed Stm. PAGE separated protein fractions on nitrocellulose were used as recall antigens after immunisation with live and heat killed Stm. This detected no qualitative differences in proteins used dominantly after live and heat-killed immunisation; however, because of the technical limitations of that system, this point is not very well answered. This leaves the possibility unresolved that, distinctly different proteins are handled during the immune response to live and heat killed Stm.

Though the qualitative T helper response is same in either \(Ily^s\) or \(Ily^f\) mice after live Stm immunisation, quantitative differences exist. It can be seen that C.D2 as well as C3H.SW mice respond relatively poorly than their \(Ily^s\) counterparts when immunized with live Stm at relatively early time points, probably because of smaller bolus of antigen persisting for continued priming as a result of faster clearance as against the growing bacteria in \(Ily^s\) mice [Fig.10 and Fig.13]. At very late time points e.g. 90 days [Fig.12] it can be seen that though, there is production of IFN-\(\gamma\) from the Stm-sonicate activated splenocytes, it is just above detectable limits of the assay. This quantitative difference does not necessarily reflect in the DTH reaction, as well as antibody titres. Studies on the similar lines as ours on \(Ily^s\) and \(Ily^f\) mice have been reported very recently. In this report, the cytokine gene expression \textit{in vivo} has
been determined after mice have been immunized with *Salmonella dublin*. The m-RNA expression, as seen by RT-PCR in liver and spleen does not differ much in *Itl* versus *It* mice, qualitatively as well as quantitatively despite the fact that bacterial counts are lower in resistant mice. This clearly indicates that *in vivo* m-RNA expression and *in vitro* cytokine determination may not match with each other (Eckmann L, et al. 1996).

It can be seen that both *It* and *It* mice of H-2b and H-2d background show high DTH when immunized with live Stm and low to undetectable DTH when immunized with heat-killed preparation pointing to Th1-like response generated [Fig.7]. Antibody isotype analysis also points towards this skewing effect, where lower anti-Stm IgG1/IgG2a ratios are seen in sera after mice have been immunized with live Stm, as compared to heat-killed Stm probably because of IFN-γ pushing the isotype switching towards IgG2a which can be seen as increased titres in live Stm immunisation [Fig.8].

*T helper responses to metabolic mutants of Stm*

From the studies on *It* and *It* mice using Stm754, it was clearly evident that there is no change in the cytokine pattern from a Th1-like response after live immunisation. As has been argued earlier, it may be due to low but continued proliferation of Stm in *It* mice. To expand on this point, we used metabolic mutants of Stm that have a block in the essential biochemical pathways. *aroA* mutant is generated by transposon insertion mutation, and the mutation is irreversible. These bacteria have a block at the shikimate synthase enzyme. As shikimate is an essential starting component in this branched pathway, it can not produce all the aromatic amino acids which are downstream to it. It so happens that aromatic amino acids are also essential amino acids for mice.
purA mutant of Stm has defect in the purine synthesis pathway and shows the growth characteristics similar to aroA mutant. Due to these reasons, the growth of live aroA mutant of Stm injected in mice is restricted to few growth cycles (O’Callaghan, et al. 1988).

It is evident from Fig. 15 that aroA mutant is not capable of replicating in vivo, as seen from splenocyte counts seen after intraperitoneal immunisation of live bacteria. At the same time, bacteria are not cleared from the body but are recoverable at all the time points checked. The persistence of bacteria without replication is not possible unless they are hidden intracellularly where their viability may be maintained, which seems to be the case here.

It can be seen from Fig. 16 that DTH reaction evoked by these immunisation is more in live aroA and purA when compared to their heat-killed counterparts. At the same time, when compared to live Stm, it is quantitatively less, though more than heat-killed Stm. This indicated to us that there can be an IFN-γ dominant response after immunisation with live metabolic mutants as against their heat-killed counterparts.

IgG1/IgG2a ratios in the mice thus immunized with live and heat-killed aroA mutant and Stm754 show that there is a decreased ratio in the sera obtained from mice immunized with live bacteria as compared to the ones from heat-killed immunized mice suggesting to us the skewed isotype switching towards IgG2a because of IFN-γ produced in mice immunized with live bacteria.

Indeed, the above effects are due to the production of IFN-γ as seen from Fig.19a. Though, quantities of IFN-γ produced by splenocytes from mice immunized with live aroA or purA mutants are lower, than those produced by
splenocytes from the mice immunized with Stm754, there is significant production of IFN-γ, as compared to their heat-killed counterparts (Fig.19). All the three immunisation with heat-killed bacteria produce IL-10 in equivalent amounts. The quantitatively less amount of IFN-γ from splenocytes of mice immunized with live metabolic mutants may be due to decreased number of bacteria and hence quantitatively less priming of T cells in those mice. This effect is seen in H2-b and H2-d mice. Increasing the dose of live aroA from $10^4$ to $10^8$ bacteria shows a quantitative increase in terms of IFN-γ as well as IL-10 produced in a T cell stimulation assay, showing that this is a result of enhanced priming in these mice due to an increase in immunisation dose of bacteria. This experiment is done at fairly late time points hence the difference in the levels of IFN-γ in $10^4$ Stm and aroA immunized mice is not distinctly visible.

It is very apparent from Fig.19b and others throughout, that splenocytes from mice immunized with live Stm754, produce high amounts of IL-10 along with high amounts of IFN-γ. This may be because the effective T cell priming that may be taking place in these mice is more because of continued replication of bacteria till the host immune system takes over completely. After the initial phase of infection, there will logically be a dynamic equilibrium between the death of bacteria and there continued replication. It may be possible that the bacteria that are getting killed, evoke IL-10 response against them, in the continued presence of IFN-γ response. It has been shown in TCR transgenic mouse model of in vitro priming of T helper cells of two different helper phenotypes, that primed Th1 cells can be driven to Th2 cells in the presence of IL-4 but Th2 cells can not be driven to Th1 responses (Perez et al. 1995), however, it is not possible to change the helper phenotypes of T cell clones. This is attributed to the possible suggestion that in polyclonal Th1 cells, there
may be cells that are still in different degree of commitment, and hence can change the phenotypes, which is not the case in case of clones.

**Comparison of T helper responses to E coli and Stm**

Stm has been shown to enter macrophages by an active process other than simple phagocytosis, through a membrane ruffling mediated endocytosis (Fransis CL, *et al.* 1993) which is sensitive to cytochalasin D. From the results obtained with *Ity* and *Ity* strains of mice, as well as metabolic mutants, it was apparent that the bacterial cell number was not a key factor in regulating the helper T cell differentiation. The next question to ask was, what is special in live bacteria that heat-killed bacteria do not have, which may be responsible for skewed helper responses. Live bacteria invade and reside inside macrophages (and many other mammalian cells such as epithelial cells, M cells etc.) (Gahring LC, *et al.* 1990), which heat-killed bacteria obviously can not do as efficiently, as they are taken up only by phagocytosis. In order to expand this point, we immunized mice with live and heat-killed preparation of a genetically related bacterium, *E coli* HB101. *E coli* and Stm share around 85-90% homology at DNA level and hence are supposed to share many housekeeping common proteins. Though enteropathogenic strains of *E coli* can invade macrophages as well as epithelial cell border, *E coli* HB101 can not do so (Fransis CL, *et al.* 1993) and can serve as a control in analyzing the importance of invasion of Stm in bringing about a skewed helper response. As it can be seen, all the strains of mice tested, show low DTH to live or heat-killed form of *E coli* whereas DTH to live Stm is distinctly higher than it's heat-killed counterpart in the same experiment, (Fig.23) suggesting that live *E coli* may not give an IFN-γ dominant response. At the same time, it was necessary to be sure that *E coli* immunizations prime T cells. As can be seen
from Fig.24, live as well as heat-killed immunisation with *E coli*, produces IL-10, whereas, levels of IFN-γ are barely detectable to non-detectable. This finding has been repeated in both H2-d as well as H2-b strains of mice. Here, the recall antigen in the assays involving immunisation with *E coli* is soluble protein extract prepared from sonicated *E coli*. It was necessary to rule out the possibility that Stm sonicate has some macromolecular complexes, that specifically initiate Th1 responses, in already Th1 driven condition, which *E coli* sonicate lacked, we did the same experiment using both the sonicates as recall antigens, as *E coli* and Stm share homology at protein as well as DNA levels. It has been shown that this does not make a difference in a readout assay of helper differentiation, ruling out this point (Fig.25). These data suggest that the capacity to invade macrophages may be necessary to trigger the IFN-γ dominant response to live bacteria. Which may be because of various factors including the processing of antigens in a different compartment producing distinctly different fragments of peptides to be loaded on MHC class II as well as initiation of transcription and translation of stress related proteins after invading the macrophages, as discussed above. Indeed the cytokine profile seen after immunisation of mice with irradiated Stm suggests this (Fig.22) where, mice immunized with, irradiated Stm which were non-heat-treated, do not produce IFN-γ. This experiment was carried out with the assumption that irradiation will not destroy the bacterial cell surface structure, as heat treatment does; hence it is assumed that such bacteria will be able to invade macrophages through a membrane ruffling endocytosis. Obviously, from our data, it is suggested that, even if irradiated Stm are taken up like live Stm, that by itself is not enough for driving an IFN-γ dominant response, and possibly one or two rounds of *in vivo* replication and/or transcription/translation are needed. Under the current condition of irradiation, our irradiation facility takes around 48 hr. to deliver 1.5 Mrad dose, that is
needed to kill live Stm. Exposure of bacteria for such a time period at room temperature may destroy the surface structures that may be needed for invasion. Similar findings have also been seen with bacteria that are killed with mercuric chloride (HgCl₂) for a short time exposure (45 min.; data not shown) stressing that active protein synthesis is necessary in vivo in order to observe IFN-γ dominant T helper response.

Ligand Density and T helper response

As has been discussed extensively in review section, there are numerous reports correlating ligand density with skewed T helper responses. It was tempting for us to analyse this point with respect to T helper development in Stm immunisation. We have, a T cell hybridoma, T21.2 that has been generated by fusion of a T cell thymoma, with spleen cells from the BALB/c J mice immunized with heat-killed Stm. This hybridoma, because of promiscuity in antigens between E coli and Stm, recognizes a shared epitope (Fig.26). Since T cell hybridomas are not known to be stringent in their co-stimulatory requirements, the extent of their activation can be directly correlated to the amount of MHC-peptide complexes present on the surface of APCs that stimulate them. We have used this model to study the MHC-peptide complexes generated after immunisation with live or heat-killed preparation of Stm754, Stm purA or E coli. It is evident from Fig.27 that the required number of APCs from Stm754 injected mice are less, to bring about equivalent extent of activation of T cell hybridoma, when compared with APCs from Stm purA and E coli injected mice. The less ligand generated may be due to lack of replication of Stm purA and E coli in vivo. Less number of APCs from Stm754 injected mice required to bring about similar level of activation of the T cell
hybridoma suggests that the net MHC-peptide ligand density generated may be high on them. High ligand density has been attributed to bring about Th1-like responses, as has been reported in the case of immunisation with human collagen IV cross-reacting peptides having differential affinity to MHC molecule (Murray JS, et al. 1992). T cell priming as well as ligand density generation is not a static phenomenon. T cell priming in vivo takes place over a period of time. Ligand density tracking over this time period in these mice, which are injected with Stm754, Stm purA as well as E coli, may shed more light. With the previous data utilizing the same hybrid (T21.2), it has been reported that APCs from mice that have been injected with live Stm754, producing high ligand density at earlier time points, drop the levels at later time points. At the same time, it has been observed that these levels are increased from initial low to a high MHC-peptide ligand levels on APCs that have been injected with heat-killed Stm754 over the increasing time period (Thatte J, Ph.D. thesis). The increased ligand density on APCs from mice immunized with live Stm754 may be a concerted effort of targeting of Stm as well as it’s replication in vivo. Now it is clear from various reports that there is initial burst of IFN-γ production from the splenocytes that are pulsed with Stm. IFN-γ is a known upregulator of surface MHC class II molecules. Whether the increased stimulation of the T cell hybridoma is due to the increase in surface MHC class II molecules, harboring the specific peptides remains to be seen. More light should be shed on the kind of APCs that are involved in this activation of T cell hybridoma. Dendritic cells are known to be important in priming naive T cells. Whether this increase in density of MHC-peptide ligand, seen with whole splenocyte population, is also true for dendritic cells remain to be seen. If true, this can suggest that the strength of signal while priming of Th0 cells leads to their skewed differentiation. Alternatively, it may be possible that priming of CD4 T cells by dendritic cells
in itself may not be responsible for its skewing phenotypically into Th1 versus Th2 cells. Variation of ligand densities generated on various other APC population may be responsible for driving this phenotypic variation of primed Th0 cells.

*Modulation of T helper responses with pharmacological agents*

It has been noted in the literature that the signaling pathways of the T helper clones are different, and that they respond to different pharmacological drugs differently. It was our aim, to find out whether the priming of T cells and their differentiation into distinct helper phenotypes is affected by these pharmacological drugs like their *in vitro* differential effect on the already committed helper T cell clones.

POF, is a drug of choice to downregulate the pro-inflammatory cytokines in various disease conditions. Along with this property, it also counteracts the effects of pro-inflammatory cytokines, such as increased oxidative bursts, degranulation, size of the phagocytic cells (Mandell GL, 1995). It has been shown to have inhibitory action on Th1-like but not Th2-like polyclonal cell populations (Rott O, *et al.* 1993). We have tried two different protocols to see the effects of this drug along with other similar drugs; a single injection as well as multiple injections at the time of priming of antigen specific T cells. As can be seen (Fig.29) single injections of POF, dbcAMP as well as CT do not seem to have any effect on the differentiation of T helper cells in our system, as seen by differential IFN-γ and IL-10 production. POF has a half life of 3-4 hr. as seen by elimination of drugs in urine (Ward A, *et al.* 1987) and may have been eliminated before its *in vivo* action. When the POF and dbcAMP are given multiple times, before the assay is done, it seems to have the effect on
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the helper cell differentiation. It can be seen that the levels of IFN–γ are increased in mice that have been immunized with live Stm in the continuous presence of POF and dbcAMP compared to the ones immunized in the absence of any drug (Fig.30a). Moreover the quantum of increase is more in dbcAMP injected mice than POF injected mice which may be because dbcAMP mimics cAMP directly, whereas POF induces the intracellular accumulation of cAMP as one of its many effects.

This effect is quantitative, that is, the response is better after priming has taken place in presence of pentoxifylline, but does not qualitatively change the T helper balance in terms of cytokine outcome. Gupta et al. (1997) have shown that in the presence of pentoxifylline, there is increased memory T cell commitment in the absence of quantitatively high priming reaction in human alloreactive assay. Similar results have been obtained in mouse alloreactive T cell assay (R. Suresh, unpublished observation). Pentoxifylline, blocks the IL-2 mRNA expression (Thanhauser A, et al. 1993), and may thereby block the bursts of primary T cells in its presence; curiously this leads to increased recall responses in alloreactive recall system discussed above. The increased recall responses in terms of cytokine production may be occurring by this mechanism.

There have been reports of POF blocking IFN–γ and overall Th1 responses in vivo (Rott O, et al. 1993) leading to prominent Th2 -like responses. It has, however, been shown that this may not be the case in alloreactive primed T cells, where T cells primed in presence or in absence of POF result in the comparable levels of IFN–γ (Gupta M, et al. 1997). Further detailed experiments involving, how this quantitative increase in IFN–γ responses in
mice immunized with live Stm affects other parameters of T helper cells have to be carried out.

_T helper responses to recombinant protein expressed in Stm_

Stm has been recently targeted to intensive study relating to a prospective recombinant vaccine delivery carrier because of availability of attenuated strains of Stm. Oral immunisation with Stm results in the mucosal as well as systemic immune response which is IFN-γ dominant upon challenge (George A., 1996). We have used an I-Eα derived epitope in C57BL/6 J mice. The epitope is in the form of a fusion protein expressed in Stm along with GST-c-myc and can be tracked with anti-c-myc antibody to check expression. This fusion protein, when expressed in Stm, can be processed and presented in the context of I-A\(^b\) molecules, when presented to C57BL/6 splenocytes as seen by the activation of specific T cell hybridoma, 1A9.F10 (Fig.32). When mice are immunized with this recombinant Stm expressing fusion protein, the responses against the Eα epitope can not be recalled _in vitro_ with synthetic peptide. It can be seen that mice are however primed with Stm, as seen by responses to Stm soluble proteins (Fig.33).

There is certain indication that this fusion protein forms the inclusion bodies inside the cytosol of bacteria (Rudensky AY, Washington School of Medicine, personal communication). It has been reported that proteins forming inclusion bodies are problematic in T cell priming (Hone _et al_. 1996) probably because these forms of recombinant proteins are highly susceptible to protease degradation by the vector, leading to quantitatively less presentation of relevant peptides in the context of class II molecules. There are reports of
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recombinant Stm expressing foreign proteins on the bacterial cell surface, embedded in outer membrane proteins, flagella or fimbriae eliciting strong T cell response (Verjans GMGM, et al. 1995; Levi R, et al. 1996; Hone DM, et al. 1996). The genetic backgrounds of mice also have been shown to play a major role in T cell priming. It has been reported there that, Stm expressing E. coli MalE protein, do not elicit the immune response in all the strains tested and this is independent of Ily status of mice (Lo-Man R, et al. 1996). The non-detectable T cell response in mice, after immunisation with Stm expressing E-α fusion protein, may be due to one of these factors. Because of our inability to detect epitope specific responses in mice immunized with Stm-Eα, further studies were abandoned. Stm expressing Eα, however remains a valuable tool to track various factors individually in TCR transgenic mice in which, all or most of the T cell express TCR recognizing Eα epitope in the context of I-Ab. This TCR transgenic mouse model of infection with Stm expressing Eα can also be used for in vitro priming using a graded doses of live or heat killed Stm754 or metabolic mutants, expressing this protein.